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Microspectrophometric Instrumentation for the Study of Pigments and Organic Molecules within Living Cells

During the period May 1, 1963 - December 31, 1963, we have been engaged in the development of new approaches to our microspectrophotometric instrumentation. Three laboratory type microspectrophotometers, M-1, M-2, and M-3 are now in various use and stages of development in obtaining spectra of living cells. Absorption spectra data have been obtained for chlorophyll and its synthesis in the chloroplasts of plant cells, for the Vitamin A aldehydes (retinene) in the retinal rods of the frog eye, for the screening pigments (ommochromes) of insect eyes, and for the hemoglobins of red blood cells. Previous reports of our progress are noted in the Ninth Annual Report of the Biophysical Research Laboratory, 1963, sent to you on June 3, 1963.

A summary of this research has recently been published in Applied Optics, 2, 899, 1963 (25 copies of which are submitted with this report). A phase of this research, "The Spectroscopy of Single Cells and Their Organelles by Microspectrophotometry," was presented at the Third Annual Meeting of the Society of Cell Biology, and the abstract has been published in J. Cell Biol., 19, 75A, 1963.

Advances in design of our recording microspectrophotometer M-3 have been made (see Fig. 1). The lens that focused the reference signal and the specimen signal of the photocell (CdS) crystal was replaced by a front surface mirror to eliminate the wavelength dependency of the focusing. The light chopper mounted between the monochromator and the

microscope was removed and incorporated in the rotating disk which alternately passes the light to the specimen and to the reference. This eliminates some artefacts introduced by the non-synchronous choppers. The aim is to have a very sensitive, rapid scan of small areas within a living cell. This means that the signal is often of the same order of magnitude as the noise. If no filtering is applied and the noise is not filtered out, the result is non-linearity, dependent on the signal to noise ratio. The calibration is therefore dependent on the intensity of the light and the wavelength, as the sensitivity of the photocell is wavelength dependent. Also, because of the long time constant of the monitoring photocell, the electrical chopping has to be accurate and constant.

If filtering is applied in the input stage, the noise will be reduced, and the electrical chopping is not critical. Because of the long time constant of the photocell, the chopping was reduced to 40 c/s. The 40 c/s content of the signal is proportional to the sum (r + s) of the reference signal (r) and the specimen signal (s). The 30 c/s content is proportional to the difference (r - s). A linear combination gives a signal proportional to the reference signal. This signal and the (r - s) signal are fed into a ratio recorder giving (1 - s/r). A disadvantage of this system is that the same amplifiers are not used for both signals. Non-linearity or change in amplification of one of the amplifiers results, therefore, in artefacts, but these disadvantages are not serious.

To improve the specimen handling, the blocking plate in the image

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plane was replaced by a concave mirror. The image on the surface of the mirror is observed via another mirror and a telescope. The concave mirror images the eyepiece in the telescope and acts as a "field lens." No aberrations are introduced by it. Accurate handling of the specimen and observation during a spectral scan are now possible.

The specimen has to be focused on the concave mirror which contains four holes. The mirror has a radius of curvature of 270 mm and is front-silvered. It is to be positioned 250 mm above the eyepiece and the two larger holes (2mm diameter) or the two smaller holes (1 mm diameter) are to be lined up with the instrument. In this way the eyepiece is just imaged in the telescope, resulting in a large field of view. The holes in the mirror are slightly tapered. The ratio of the amounts of light coming through these holes and from the specimen imaged on the holes is then determined. To do this, a light chopper, a rotating disk (10 revolutions per sec.) was incorporated. It has two open segments of 45° separated by a closed segment of 45° and a closed segment of 235°. In this way, 40 flashes per second may fall upon the photocell, i.e., two flashes from one hole followed by two flashes originating from the other hole.

The use of the instrument in its present state is limited by the photocell (CdS) properties. Different photocells have different characteristics; for instance, the sensitivity may differ from one photocell to another, and changes with time. The sensitivity drops about 20 times from 730 to 400 mμ, and the time constant increases. These effects are enhanced by the use of a tungsten light source which has a drop of about 5 times over that range. As a result we are now investigating various

photomultiplier tubes, new light sources, and the use of an automatic gain control.

The 40 c/s content of the photocell signal and the 30 c/s content together submit sufficient information for computation of the absorption ratio. Therefore, the signal is fed into two filters after initial amplification (80x) and filtering (fo = 34.6 c/s, 60%). One of them is tuned to 40 c/s, the other to 30 c/s, both having a Q value of 40 and a gain of 80. The output signals of the filters are rectified, added, and smoothed (T = 4 sec). The signal fed into the 40 c/s filter can be reduced with help of a potentiometer. This potentiometer is set so that the sum signal is equal to the reference signal and is independent of the amount of light passing the specimen hole. The output of the 30 c/s signal is proportional to the difference between reference and specimen signal. This signal is also rectified and smoothed (T = 4 sec), and fed, together with the reference signal, into a Varian recorder via a span potentiometer.

The recorder is used as a ratio recorder, plotting $(1 - \frac{I}{I_o})$, one minus the ratio of specimen and reference signals. The performance is determined by the sample size, wavelength selectivity, and scan velocity. The spectral range from 400 to 650 mμ is easily and accurately obtained for a sample of 4 μ diameter, a wavelength half-band width of 5 mμ with a scanning speed of 1.3 mμ/sec.

A more complete report of the instrument M-3, its electronic design, capabilities, and performance will be summarized in a later report.

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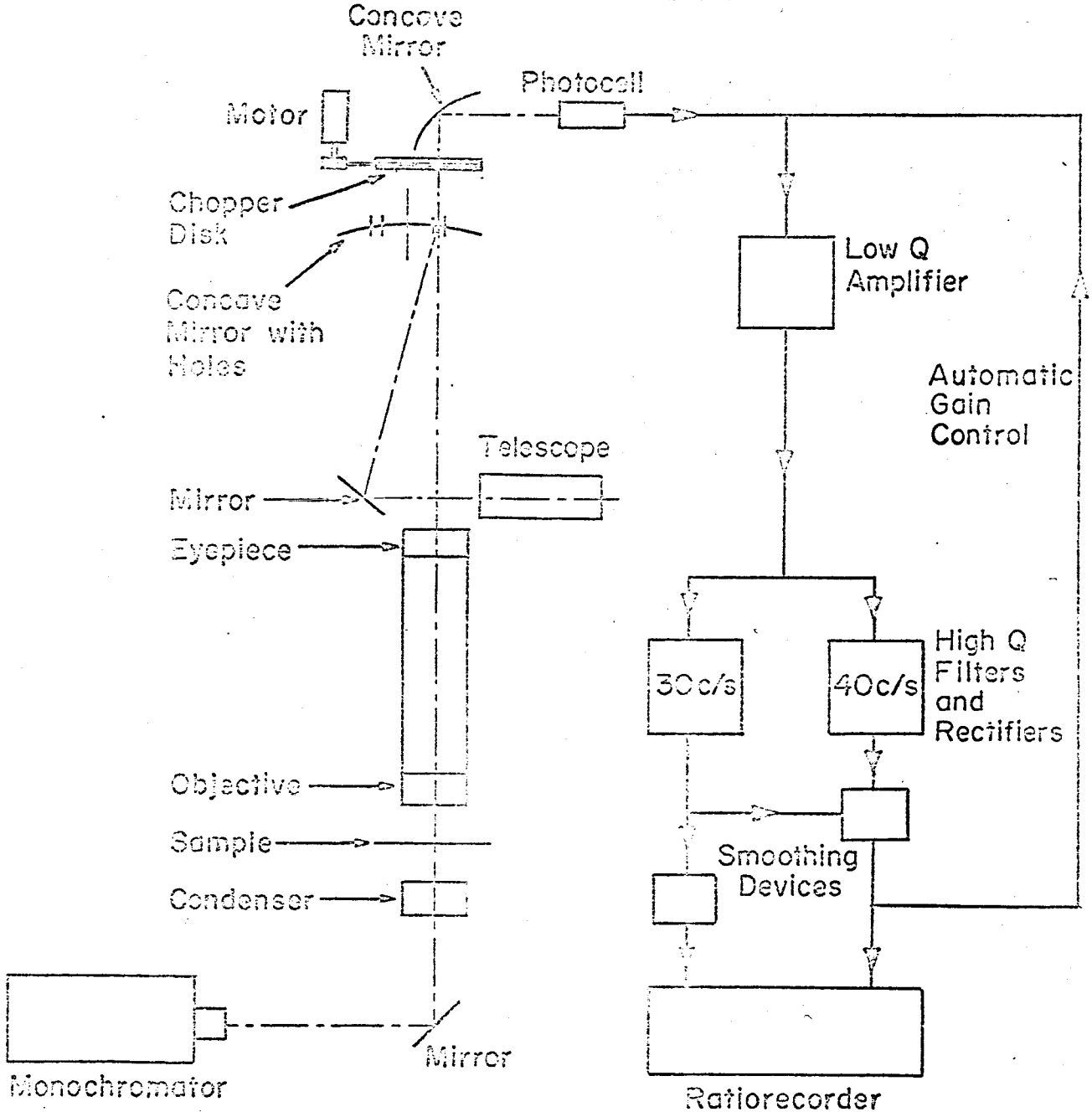

FIGURE 1